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(54) Title: ENHANCEMENT OF LEARNING AND/OR MEMORY BY A HUMAN SOMATOTROPIN

(57) Abstract

The ability of an individual to learn and remember is improved by administering somatotropin to the individual before the task or information is first taught or presented to the individual. The present invention is the use of somatotropin (growth hormone) and related compounds for enhancing the mental abilities of normal, defective and deficient individual. Somatotropin is particularly useful for people with brain injury, mental retardation, and degenerative diseases such as Alzheimer's disease.

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ENHANCEMENT OF LEARNING AND/OR MEMORY BY A HUMAN SOMATOTROPIN

FIELD OF THE INVENTION

The invention relates to compounds and compositions which improve the learning and memory ability of a human or animal.

BACKGROUND TO THE INVENTION

Throughout the years, many scientists have attempted to improve the learning and memory of both normal and learning or memory deficient or impaired people. The methods tried include exercise, an assortment of mechanical devices, puzzles and other techniques to practice using mental skills, certain foods, e.g., coffee, fish and other supposed "brain food", surgical procedures and drugs.

Within the category of drugs, many different compounds have been tested with varying degrees of success. These include different types of steroids such as glucocorticoid (cortisol, corticosterone and dexamethasone), mineralocorticoid (aldosterone) and androgens (dehydroepiandrosterone, dehydroepiandrosterone sulfate, androstenedione, testosterone, dihydrotestosterone, androgen precursors such as pregnenolone and pregnenolone sulfate). However, these drugs have many side effects, some of which may outweigh their benefit, a small improvement in learning and memory. For example, glucocorticoid have the following side effects: hyperglycemia, hyperlipidemia, obesity, predisposition to diabetes mellitus, hypertension, hirsutism, protein catabolism, muscle wasting muscle weakness and osteoporosis. It should be readily apparent that almost all of these side effects are particularly undesirable in the aged population, the very group in greatest need of learning and memory enhancement. Mineralocorticoid can cause sodium retention, hypervolemia, edema, hypertension, potassium depletion, hypokalemia and its sequela such as alkalosis and cardiac arrhythmias. These side effects are also particularly undesirable in the

aged. The use of androgens may result in precocious puberty, virilism and disturbances in reproductive function.

A number of nonsteroidal drugs have been used with some success, including adrenocorticotropic hormone (ACTH) and its analogues, vasopressin, opioid antagonists, neurotrophic or nerve growth factors, various neurotransmitters and neuropeptides, their inducers and modifiers, CNS acting stimulants, antidepressants, anxiolytics, nootropics and vasodialators. Morley, Journal of the American Geriatrics Society, 34: 52-62 (1986), Hock, Neuropsychobiology, 17, 145-160 (1987), Weizman et al, Life Sciences, 40: 2247-2252 (1987) and Crook, Seminars in Neurology, 9(1): 20-30 (1989). However, all of these have side effects which are summarized below:

ACTH: sodium and fluid retention, muscle weakness, hyperpigmentation, hypertension, suppression of growth in children, and negative nitrogen balance.

Vasopressin: anaphylaxis, cardiac arrest, nausea and vomiting, vertigo, and bronchial constriction.

Opioid antagonists (e.g., naloxone): hypotension, ventricular tachycardia and fibrillation, and pulmonary edema.

Nerve growth factors: no information re side effects.

Neurotransmitters (e.g., epinephrine): anxiety, weakness, dizziness, cardiac arrhythmias and cerebral hemorrhage.

CNS stimulants (e.g., amphetamine type sympathomimetic amines): elevation of blood pressure, tachycardia, dizziness, insomnia, diarrhea, impotence and changed in libido, and suppression of growth in children.

Antidepressants (e.g., monoamine oxidase inhibitors): hallucinations and convulsions, insomnia,

hypotension and hypertension, dizziness and vertigo, fatigue, and inhibition of ejaculation.

Anxiolytics (e.g., benzodiazepines): drowsiness, ataxia, skin rash, nausea, impairment of sexual function, and vertigo.

Nootropics (e.g., dihydroergotoxine): nausea and vomiting, weakness and muscle pain, tachycardia or bradycardia, and precordial distress and pain.

The degree of success with these drugs has not been sufficient for the use to become generally accepted.

The mechanisms of learning and memory is not understood. Several competing theories exist, none of which seems to explain the entire process. Lynch et al, Science, 224: p. 1057-63 (1984). Thus, theory does not provide much help in finding a suitable treatment. To complicate matters even more, a number of charlatans have promoted a number of bogus "miracle cures" based on anecdotal evidence and uncontrolled experiments. Somatotropin, also called growth hormone, is a natural protein secreted by the pituitary gland. In humans this protein consists of 191 amino acids and has a molecular weight of about 21,500 in its mature form.

Somatotropin has been used to treat hypopituitary dwarfism in humans. This protein was first produced by laborious extraction from the pituitary glands of cadavers. The amount recovered was not adequate to meet the demand for treating dwarfism. Next, human pituitary cells were cultivated as a more convenient source. However, this approach was abandoned as a result of the risk of Creuzbold-Jacob syndrome, which is caused by a slow virus inhabiting human pituitary cells. Furthermore, the gene for somatotropin has been cloned and expressed to produce large quantities of the protein (or of Met-hGH) in nonhuman cell

culture. See U.S. patents 4,342,832, 4,521,409 and 4,895,600.

More recently, transgenic animals have been produced with a somatotropin transgene. When expressed, the transgene has been shown to cause the animals to grow faster, produce a greater meat to fat ratio and, in the case of dwarf breeds, oppose the tendency to dwarfism.

Somatotropin has also been used to treat burns, wounds, polio, dystrophy, bone knitting, diffuse gastric bleeding, emphysema and pseudarthrosis in humans, and for enhancing mammary development, milk production and increasing meat while reducing fat in animals.

The amount of somatotropin circulating in the blood is affected by a large number of physical conditions (hypoglycemia, fasting, exercise, trauma, blood loss, response to histamine, vasopressin, pyrogen, amino acids, etc.) and psychological conditions (such as stress). Mason et al, Psychosomatic Medicine, 30(5): 760-773 (1968). Increased amounts of somatotropin has been shown to be produced by animals in response to electric shocks. When the animal learned to avoid the shocks, serum levels returned to normal. Feldmann et al, Psychoneuroendocrinology, 1: 231-242 (1976). Reduced amounts of somatotropin are released in older animals. Sonntag, Endocrinology, 107(6): 1875-1879 (1980).

The effect of endogenous and exogenous growth hormones on memory has been sharply disputed.

According to Mosier, et al., Pediatrics, 36 (suppl.): 465-519 (1965) (Mosier I), in humans, shortness of stature is associated with mental retardation. However, this conclusion has been challenged by Meyer-Bahlburg et al., Psychological Medicine, 9:187-189 (1979). While, in young rats, induced brain lesions which produced a generalized learning deficit also impaired growth, Mosier,

et al., Pediatric Res., 27(2):181-185 (1990) (Mosier II), Mosier excludes hypopituitarism (hence, reduced secretion of GH) as a cause of the learning defect (page 184). He acknowledged that the animals "have normal growth hormone function" (abstract).

More positive evidence of a GH effect on memory is found in the work of Hoddes et al. and Noguchi et al. Hoddes, Sleep 1(3); 287-297 (1979) discloses altering the memory of mice by injecting bovine growth hormone into mice shortly before and shortly after the original learning run. Only one dose was given. The earliest before original learning was 90 minutes. Better memory was shown when the drug was administered 90 minutes before or 30 minutes after but worse memory when it was given around the time of original learning. Noguchi et al., Journal of Neurochemistry, 38(1): 246-256 (1982), reported that in comparison with normal controls, hydrocortisone-intoxicated neonatal rats had smaller cerebra, lower CNPase activity, and greatly reduced learning ability. They suggested that the hydrocortisone stunted cerebral development. Since electron microscope observations revealed that the GH cells of the anterior pituitary glands of the HC rats possessed many more secretory granules, they inferred that the HC rats might have a disorder in the system that releases GH from the secretory cells. They therefore explored whether administration of bovine growth hormone to neonatal HC rats would have a restorative effect. The bGH treatment partially restored cerebral size and DNA content, CNPase activity, and learning ability in the HC rats.

However, Noguchi's findings are not readily extrapolated to other situations, as the effects of hydrocortisone and other steroids on learning and memory are complex. Tamasy, et al., Physiol. Behav., 10:995-1005 (1973) found that hydrocortisone improved passive-avoidance

learning in both normal and adrenalectomized three month old male rats. Cottrel and Nakajima, Pharmacol. Biochem. & Behav., 7:277-280 (1977) reported that hydrocortisone restored the avoidance response deficit in cycloheximide treated rats. Nakajima, Physiol. Behavior, 20:607-611 (1978) revealed that hydrocortisone attenuated retrograde amnesia, produced by electroconvulsive shock, in the mouse. Beckwith, et al., Physiol. Behav., 36:283-286 (1986) found that early recall of word lists by male undergraduates were improved by hydrocortisone. Late recall was facilitated by a high HC dose and impaired by a low dose. See also Wolkowitz, et al., Am. J. Psychiatry, 147:1297-1303 (1990).

The present invention relates to the use of growth hormone to improve learning and memory in humans or other animals which are not affected by treatment with hydrocortisone or other steroids.

Even if Noguchi's findings were to be given a broader currency, there are several contradictory reports, such as those of Gold (1976) and Meyer (1979). Gold et al., Hormones and Behavior, 7: 509-517 (1976), disclose that growth hormone had no effect on memory retention when administered immediately post trial, whereas ACTH, epinephrine and norepinephrine improved memory retention relative to saline controls. Meyer et al., Physiological Medicine, 9: 187-189 (1979), acknowledges a debate among researchers concerning the link between growth hormone and learning. The earlier data reviewed by Meyer suggested that growth hormone administered to pregnant rats improved learning behavior in the offspring. The data presented in the Meyer paper indicated that low growth hormone levels was not associated with mental retardation in humans.

Besides somatotropin, other peptides which functionally are part of the somatotropinergic system, such as growth hormone-releasing factor and somatostatin, have

also been suggested to have an effect on memory. Alvarez et al, Meth. Find. Exp. Clin. Pharmacol., 12(7): 493-499 (1990), disclose the treatment of humans with growth hormone releasing factor (GRF) resulted in improved memory scores. The dosage was administered two hours before testing. The reference also cites earlier work showing marked improvement in mental performance, social behavior and school activities in children with short stature under treatment with GRF. This earlier data may be discounted as better adjusted children who are overcoming a stigmatizing physical feature (shortness) would be expected to do better without any direct effect of the drug on school activities. Moreover, Alvarez, et al. suggests that the effect is attributable (see page 498) to GRF's neurotransmitter activity, rather than to its regulation of GH.

Cacabelos et al., Hormone Research, 29: 129-132 (1988), disclose that a single dose of GRF administered to Alzheimer Disease patients with early onset, pre-stage IV SDAT resulted in transient improvement in mental performance. There was no improvement in late onset SDAT patients with severe dementia. While Cacabelos et al. report somatostatin and GRF are antagonistic in behavioral effects, both partially improve learning abilities, with SS being more effective. Since SS and GRF were both effective, despite their antagonistic effects on GH levels, it is unlikely that their enhancement of learning is attributable to their regulation of GH. Somatostatin decreases secretion and plasma levels of GH.

Vila, U.S. Patent 5,089,472, teaches administration of GRF to improve the attention or short term memory of a subject.

Crook, Seminars in Neurology, 9(1): 20-30 (1989), discloses that Alzheimer's disease patients have lower levels of somatostatin (which inhibits release of growth

hormone) in the brain. However, he reports that administrating a somatostatin agonist analog did not have any significant effect.

Accordingly, the relationship between somatotropin and learning and memory, if any, is contradictory and unclear. While clinicians have used somatotropin for years to treat physical conditions, the psychological aspects remain relatively unexplored. Additionally, due to inherent problems with the testing appropriate to test such an association, incidental observations are not properly controlled and subject to challenge.

Human growth hormone has not previously been used to improve learning and memory.

SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the deficiencies in the background art while enhancing learning and memory in individuals.

Learning and memory are improved by long term administration of an effective amount of human somatotropin, to the subject.

The GH may be used to ameliorate the effects of a disease or injury which causes a reduced ability to learn and remember. For example, a GH may be used to prevent and/or reverse the memory deterioration associated with Alzheimer's disease, of to aid in the recovery of a patient whose brain has been injured, such as by trauma, stroke, oxygen deprivation, infectious agents or toxic chemicals.

Somatotropin also may be used to enhance learning and memory when no deficit initially exists.

Another object of the invention is to provide means for testing the effects of other peptide or protein drugs in animals, without unduly stressing the animals.

This is accomplished by use of transgenic animals engineered to produce the drug, as was done herein for human somatotropin.

BRIEF DESCRIPTION OF THE DRAWINGS

Graphical representations of the results from the experiments are shown as Figures 1-3.

Figure 1 compares the rate and degree of learning over time for the treated group compared to controls.

Figure 2 compares the degree of retention of learned knowledge as tested at a later time for both the treated and the control group.

Figure 3 compares the rate and degree of learning over time for the treated and control groups.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions of learning and memory

Learning and memory are processes by which organisms adapt to their changing environments and retain the effects of experience into the future. Learning is not easily defined since it is a process which must be inferred from changes in behavior. Such changes in behavior are not the result of temporary states such as fatigue, drug alternations or motivational factors (hunger, etc.) or of normal developmental alternations. Learning is usually the result of some experience or practice. On the other hand, memory is usually defined as the result of the learning process and is the means by which the knowledge gained is stored either temporarily or permanently. Obviously, some form of memory must be present in order to infer that learning has taken place, since without memory, there could be no observed changes in subsequent behavior (see, e.g., Hill, W.F. *Learning* Harper and Row, 1990)

Learning can be defined by the type of process which produced the change in behavior. Thus, various operational definitions of learning exist, such as classical conditioning (pairing of two stimuli), instrumental conditioning (making a stimulus or reinforcement contingent on a response), verbal learning (learning words and meanings), latent learning (learning by observation without overt action) and many more, depending on the operations used. Learning can also be classified much as can memory (see below) by type of experience, such as motor, sensory, cognitive and so forth.

In humans, memory is usually separated into short-term and long-term memory. The shortest memory store is the sensory memory which lasts on the order of 1/2 to 2 or 3 seconds. This is the memory of a scene which is retained after one glances at it and looks away. Regular short-term memory is that memory which occurs after a sensory experience, such as seeing an event or feeling a stimulus, but which lasts only a relatively short time, on the order of 2-20 seconds. Short-term memory is a limited store, usually found able to store 7 plus/minus 2 chunks of data. Short-term memory is thought to be a basically electrical event or some reversible change in synaptic process but which begins to fade rapidly.

Long-term memory is the fairly permanent storage of learning in an apparently infinite capacity store which is thought to entail some change in brain structure or chemistry. Long-term memory can be further subdivided into several broad categories, depending on content or type. Declarative memories are those memories of facts which are available to the conscious process. Declarative memory can be further subdivided into semantic memory those memories of facts about the world, and episodic memories, those facts about oneself, which are not common to other people. There

is evidence that each of these memory systems are subserved by somewhat distinct but overlapping brain systems (see e.g., Squire, Knolton and Musen. The structure and organization of memory. *Annual Review of Psychology*, 1993, 44, 453-495). Procedural (sometimes known as non-declarative memory) memories are skills and automatic operations which are not stored with respect to specific times or places.

Deficits in learning and memory

Deficits in learning and memory are hard to differentiate since if there is no memory formation, one cannot tell whether or not learning occurred. Thus, often, deficits in learning may mean that the subject simply cannot form appropriate memories or that some deficit in the cognitive process preclude actual learning. Learning disorders may be caused by abnormal development, early drug use or exposure, and other factors which impede the ability of the organism to focus attention on a situation or understand the context or meaning of the sensory experiences. Problems in memory itself are usually caused by head trauma, vascular disorders, infections, and degenerative or developmental processes. The type of memory affected will depend on the area of the brain involved. Problems with the motor regions of the brain, such as the cerebellum and certain regions of the basal ganglia, will produce deficits in motor learning and memory with automatic motor tasks affected. Degeneration of the temporal lobes will produce deficits in recognition of various types for both semantic and episodic memories. Hippocampal damage will produce primarily problems with long-term memory storage and retrieval. Degeneration of the cortex can cause various memory and/or attentional problems, ranging from loss of episodic memory or loss of semantic memory, to the

inability to connect various memories with immediate sensory experience. Obviously, trauma, infection or degeneration can strike any region, thus causing associated learning and/or memory alternations of deficits (See Kolb and Whishaw, *Fundamentals of Human Neuropsychology*, Freeman, 1990).

Human tests for learning and memory

There are many tests for human learning and memory. Verbal short-term memory is often tested by digit span tasks in which the individual is exposed to numbers containing various digits for various times and asked to recall the digits some time later. Nonverbal short-term memory can be tested by various motor or spatial memory tasks, such spatial information tests. (Cave and Squire, *Hippocampus*, 2:151-163). In these tasks, the subject is exposed to various motor tasks or spatial orientations and asked to recall or reconstruct them later.

Procedural memory is that memory underlying motor performance or skills. In humans, the separation of procedural and declarative memory is not a simple task, because the human may develop declarative memory strategies for motor performances. However, this type of memory can be tested with various tasks such as mirror drawing tests, mirror reading tasks, weight sampling tasks, speed reading of repeated nonwords, and resolving random-dot stereograms (see, e.g., Cohen and Squire, *Science*, 210,207-210, 1980; Musen and Squire, Neuropsychology. *Journal of Psychology, Learning Memory and Cognition*, 20, 441-448, 1991: Benzing and Squire, *Behavioral Neuroscience*, 103, 548-560, 1980). In addition, various forms of classical conditioning tasks, such as eyeblink conditioning in which a tone or light onset is paired with an air puff to the eye can be used to test procedural memory function. This type of task has many

forms and variations which have been used on various deficits and conditions. Many other tests of human procedural memory exist, such as those used to test classification abilities which are included in procedural memory. Here, subjects are tested on ability to classify letter strings as grammatical or nongrammatical, among other tests (Knowlton, Ramus and Squire, *Psychological Science, 3,* 172-179, 1992). To list all such tests would be almost impossible due to number and variety. However, among other reviews, a recent article by Squire and his associates gives a good overview (Squire, Knowlton and Musen, *Annual Review of Psychology*, 44, 453-495, 1993).

Declarative memory in both forms can be tested with many sorts of tests, including fact recall, matching tests of various sorts, tests for retention from minutes to days or years, verbal learning and recall tasks, and many other such tests. Here the number and variety of tests is very great and depend on whether the deficits is thought to be in autobiographical (episodic) or world facts (semantic) memory systems. Refer to Squire, Knowlton, and Musen, *Annual Review of Psychology*, 44, 453-495, 1993, and Kolb and Whishaw, *Fundamentals of Human Neuropsychology*, Freeman, 1990 and Raaijmakers and Shiffrin, *Annual Review of Psychology*, 43, 205-234 for overviews.

Animal models for learning and memory

There are many animal models for investigating learning and memory. Any of these can also be used for testing deficits in these processes. These models range from very simple forms of animals to animals thought to be close to humans in many functions, such as chimpanzees. Among the most simple animal models are the well developed paradigms for learning and memory in such primitive invertebrates as the sea slugs *Aplysia* and *Hermissinda*. In

these models, simple classical conditioning tasks or odor preference training are used to assess the ability of the animal to learn and remember (see Hoitblat and von Fersen, Comparative Cognition; Representative and Processes in Learning and Memory, *Annual Review or Psychology*, 43, 671-710, 1992 for a review). Mice and rats are also favorite models for learning and memory tests. In these animals, various types of maze tasks or operant conditioning tests are used to assess both procedural and associative learning and memories. Rabbits are widely used for classical conditioning tests, assessing various measures of associative learning and memory (e.g., Port and Patterson, Sensoryu preconditioning in the rabbit following ACTH injections, *Physiology and Behavior*, 35, 443-445, 1985). Other animals such as pigs, gerbils, various bird species, and many others are used in various types of tasks for learning and memory studies, primarily for procedural or associate learning and memory tasks. Higher mammals, such as monkeys and chimps are also tested, although more for higher mental functions such as discriminations and matching to sample tasks(see, e.g. Richardson-Klavehn and Bjork, Measures of Memory, *Annual Review of Psychology*, 39, 475-543, 1988; Spear, Miller, and Jagielo, Animal Memory and Learning *Annual Review of Psychology*, 41, 169-211, 1990 for reviews and citations). Obviously, procedures such as brain lesions, chemical interventions and other life-impairing interventions can be carried out in these animal models which are not possible in humans.

Procedures for testing animal learning and memory

The number of procedures used for testing animal learning and memory is almost as large as the list of animals used for assessing these processes. Perhaps the most basic test is the classical conditioning paradigm in

which a stimulus which produces little or no response in the response system being tested is paired with a stimulus which reliably produces the response or responses. With continued pairing, the animal learns to anticipate the second stimulus with a response to the first stimulus. Many variations on this theme are used to make the task more complex and difficult, including various time and intensity variations, pairing contingencies and so forth (Lavond, Kim and Thompson, Mammalian Brain Substrates of Classical Conditioning, *Annual Review of Psychology*, 44, 317-342, 1993).

Maze tasks are another frequently used procedure in animal learning and memory tests. Various types of maze from simple T mazes to very complex and specialized mazes have been developed. Simple step down tasks, water mazes and puzzle box tasks are also used. With these tasks, various levels of complexity and cues for learning and memory can be easily varied, such as color, type of turn, olfactory cues, reward type, pattern recognition, various discrimination types and so forth can be varied.

Operant conditioning is another widely used procedure. In this technique, a behavior is selected and reinforced either positively or negatively, or is punished when it occurs. Again, many variations exist to test for various aspects of learning and memory, including ability to judge time, predict numbers of occurrences, and many other functions. With operant conditioning, animals can be induced to learn to perform very complicated series of behaviors which tax the ability of the organism in several dimensions.

In discrimination tasks, animals must learn and remember various cues to attain reward or escape punishment. Almost all sensory modalities can be used for discriminative functions and cross modal discriminations and

generalizations are often employed. (see Spear, Miller and Jagielo, Animal Memory and Learning, *Annual Review of Psychology*, 41, 169-211, 1990 for an overview).

With higher order species, such as monkeys and chimps, many variations of the above procedures can be used to take advantage of the ability of these animals to process higher order symbols. In addition, in higher chimps, techniques for assessing the ability of these animals to form verbal learning have been developed which take advantage of the fact that these animals have cognitive verbal ability of approximately a two year old human infant.

Normal and abnormal values in learning and memory

In assessing any measure of learning or memory, the data from the experimental or manipulated group must be referred to the performance of a standard group, which may be a particular patient population, or a "normal" population. In some cases, this can be a within subject comparison in which an organism's performance after an intervention is referred to the preintervention performance. In other circumstances two or more separate but otherwise equal groups are compared, with the intervention being the difference. In some cases, individual performance rather than group performance is used, with the comparisons usually being overtime or task rather than between individuals. Whatever the measure being used, the manipulated group's performance must be tested with statistical analysis to determine if the manipulation produced a performance which was significantly different from the nonmanipulated group. Differences in absolute scores, variability around the mean score and mean score differences, among other measures, are used to determine the effects of the manipulation. (For the purpose of the present invention, a difference is deemed "statistically significant" if it is significant at the 0.05

level). It must be assumed that the performance of the standard group, including the variability, numbers of responses, mean scores and other measures such as latencies, amplitudes and speed of responding are the normal performance characteristics of the group. Various analyses exist for determining both the values of the normal group and for assessing whether the manipulated group differs significantly from normal. These tests include t-tests, analyses of variance, multivariate analysis of variance, chi-square and so forth. In many cases, with the procedures and models mentioned above, enough is known about the learning and memory process that the normal values are available. However, in any case, normal is assumed to be the group average, including variability around the average, and substantial deviations from those values, e.g., of one, two or three standard deviations, will be considered abnormal. The means and standard deviation may vary depending on the population, e.g., males vs. females.

Any of the above animal or human models may be used to tests for the learning and memory effects of a human Somatropin as described herein. A human somatotropin is considered to have an effect on learning and memory if a statistically significant effect is observable in a human or animal (whether or nor one of the models mentioned above) when some form of learning or memory is tested, including but not limited to testing by one or more of the test described above.

Transgenic Animal Models

To adequately establish any effect of a drug on mental functions such as learning and memory, one must first remove other factors. Previous attempts to measure learning and memory may be flawed in failing to account for the stress induced by administering the drug. Therefore, until

one has a properly designed screening and testing system, one does not have the ability to adequately demonstrate what compounds will be an effective drug for this purpose.

Stress is well known to affect mental functions. Among other factors, stress causes the release of quantities of various neurotransmitters and steroid hormones. A number of neurotransmitters and steroids have been known to affect mental functions such as learning and memory. One well known stress related compound is ACTH which has been shown in many studies to affect learning and memory. For a review, see Hock, "Drug Influences on Learning and Memory in Aged Animals and Humans", Neuropsychobiology, 17: 145-160 (1987).

From the standpoint of a mouse, a human grabbing it by the tail resembles a very large predator about to kill and eat it. The act of injecting a substance may appear to resemble the teeth of a giant predator biting the mouse. It is little wonder that picking up a mouse and injecting it with a needle causes stress in the animal.

The shock and pain of injecting a potential memory or learning-enhancing drug, especially on a regular basis for extended periods of time, may interfere with the results because injections to an animal cause stress. Thus there is a need for an animal model which avoids this interference.

Thus, in one embodiment, the present invention relates to a method of screening protein (incl. peptide) drugs for memory or learning-improving activity, wherein the drug is not injected into the animal. This method is especially useful for drugs which are not efficiently delivered by oral administration. In this method, a gene encoding the drug, or a protein (e.g., an enzyme or a prodrug whose expression results in the metabolic production of the drug, is expressed in a transgenic animal. Preferably, expression is subject to regulation by operably

linking the gene to a regulatable promoter especially one which, like the cytosolic phosphoenolpyruvate carboxykinase (PEPCK) promoter, is readily regulated by dietary modification or other nonstressful means.

Once a suitable drug is identified in this transgenic animal model, it may be administered to a human.

Human Somatotropin

In another embodiment, the present invention relates to the use of a human somatotropin (growth hormone) to improve learning and memory in a normal or impaired human or other animal subject.

The term "a human somatotropin", as used in this application, is intended to include both wild-type human somatotropin, and homologues which retain at least 10% of a favorable learning and/or memory-affecting activity of wild-type human somatotropin, and which have an amino acid sequence which, when aligned with that of wild-type human somatotropin in a manner conventional in the art, have a sequence similarity of at least 85% more preferably at least 90%, still more preferably at least 95%, which similarity is achieved by a statistically significant alignment.

Preferably, all, or substantially all, of the mismatches fall into the following categories:

(a) insertions or deletions at the amino or carboxy termini of wild type human somatotropin;

(b) insertions or deletions at residues outside domains GD1-GD5 as defined by Watahiki, et al., J. Biol. Chem., 264:312-316 (1986),

(c) conservative substitutions of amino acid residues, especially (though not necessarily) of residues outside domains GD1-GD5.

Abdel-Meguid, et al. (1987) determined the 3D-structure of recombinant methionyl porcine growth hormone, and suggested that it revealed the "general three-dimensional fold" of the growth hormones. The 3-D structure can be used to identify interior and surface residues; generally speaking, proteins mutated at surface residues (other than the receptor binding site) are more likely to remain functional. However, Creighton and Chothia, Nature, 339:14 (1989) discuss the toleration of mutations at buried residues. The 3D structure may also be used to determine the location of flexible surface "loops"; proteins are more tolerant of deletions and insertions in such regions.

Cunningham, et al. (1989) used homolog-scanning mutagenesis to identify the epitopes of hGH for its cloned liver receptor. Only variant hormones having mutations in regions C(54-74), F(164-190), and, to a lesser extent, A(11-33) exhibited reduced binding affinity. Cunningham and Wells, Science, 244:1081 (1989) used a related technique, alanine-scanning mutagenesis, to further study these regions.

In terms of the kinds of substitutions which may be made, one may look first to analyses of the normalized frequencies of amino acid changes between homologous proteins of different organisms, such as those presented in Table 1-2 of Schulz and Schimer, supra and Figure 3-9 of Creighton, supra. Based on such analyses, we define conservative substitutions as exchanges within the groups set forth below:

I small aliphatic, nonpolar or slightly polar residues -Ala, Ser, Thr (Pro, Gly)

II negatively charged residues and their amides Asn Asp Glu Gln

III positively charged residues - His Arg Lys

IV large aliphatic nonpolar residues -

Met Leu Ile Val (Cys)
V large aromatic residues -
Phe Tyr Trp

Three residues are parenthesized because of their special roles in protein architecture. Gly is the only residue without a side chain and therefore imparts flexibility to the chain. Pro has an unusual geometry which tightly constrains the chain. Cys can participate in disulfide bonds which hold proteins into a particular folding; the four cysteines of bGH are highly conserved. Note that Schulz and Schimer would merge I and II above. Note also that Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, etc.

Watahiki, et al. (1989) compared the sequences of flounder, yellowtail, tuna, salmon, chicken, rat, porcine, ovine, bovine and human growth hormones. Table 1 below shows the number of times each of the twenty amino acids occurs in wild-type hGH and the number of times that one of the nine other GHs discussed by Watahiki had a different amino acid at the homologous position. Thus, there were seven occurrences of Ala (A) in hGH. If there were complete divergence, the second column would have the entry 63 (7 x 9). Instead, the entry is 28.

Table 2 analyzes the substitutions in greater detail. Each row is an amino acid in hGH. Each column is a substituted amino acid in a compared growth hormone. The sum of the row entries in Table 2 should equal the value in the second column for the corresponding row in Table 1. Thus, phenylalanine (F), while conventionally placed in exchange group V (exchanges with Tyrosine (Y) and Tryptophan (W)), actually is more freely replaced in the growth hormone family. It is replaced in the following order: Leu > Tyr > (Asn = Gln)> Ala > (Gly = Ile = Ser = deletion) > (His = Lys = Thr). Bear in mind, however, that this is unnormalized

data; tryptophan is a rare amino acid (occurs only once in hGH). The term "natural substitution" shall denote any substitution of a residue in wild-type hGH which is found in nature in a corresponding residue of another vertebrate growth hormone.

Site specific mutagenesis may be used to alter one or more of the protein's activities or to improve the production and purification of the protein. One such example is to remove one or more internal methionine residues to be able to separate and purify the protein by cyanogen bromide cleavage of a fusion protein. Other examples include to removing a cysteine to prevent the formation of disulfide bridges in the secondary structure of the protein, alter the active site to enhance its activity or to change or destroy an active site to prevent an unwanted side effect.

Covalent modifications of the peptide are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. Examples include glycosylation, acylation, terminal amide moieties, etc.

Pharmaceutical Compositions and Methods

The present invention also provides pharmaceutical compositions and formulations for improving memory and/or learning in humans and animals. The invention can be incorporated in conventional solid or liquid pharmaceutical formulations in any concentration desired. For example, injectable, adsorption through the mucosa and transdermal administrable solutions may be used for treating mammals that would benefit from such treatments. The pharmaceutical formulations of the invention comprise an effective amount

of the somatotropin, its analogue or compound causing its release as the active ingredients. Other active or inert ingredients may be added.

In addition to the pharmacologically active compounds, the new pharmaceutical preparations may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Preferably, the preparations, particularly those preparations which can be administered orally and which can be used for the preferred type of administration, such as tablets, dragees, and capsules, and also preparations which can be administered rectally, such as suppositories, as well as suitable solutions for administration by injection or orally, contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound(s), together with the excipient.

Suitable excipients are, in particular, fillers such as saccharides, for example, lactose or sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example, tricalcium phosphate or calcium hydrogen phosphate. polyethylene glycol. Dragee cores are provided with suitable coatings which, if desired, are resistant to gastric juices. For this purpose, concentrated saccharide solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, polyethylene glycol and/or titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. In order to produce coatings resistant to gastric juices, solutions of suitable cellulose preparations such as acetylcellulose phthalate or hydroxypropymethyl-cellulose phthalate are used. Dye stuffs or pigments may be added to the preparation, for example, for identification or in order to characterize combinations of active compound doses.

Possible pharmaceutical preparations which can be used rectally include, for example, suppositories which consist of a combination of the active compounds with a suppository base. Suitable suppository bases are, for example, natural or synthetic triglycerides, or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the active compounds with a base. Possible base materials include, for example, liquid triglycerides, polyethylene glycols, or paraffin hydrocarbons.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspensions of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The pharmaceutical formulation for systemic administration according to the invention may be formulated for internal, parenteral or topical administration. Indeed, all three types of formulation may be used simultaneously to achieve systemic administration of the active ingredient.

For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, or buccal routes. The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

The somatotropin may also be administered in the form of an implant. The implant should have a delayed release of the drug due to delayed solubility, slowly degrading carrier, or a osmotic or other pump.

Suitable formulations for topical administration include creams, gels, jellies, mucilages, pastes and ointments. The compounds may also be formulated for transdermal administration, for example, in the form of transdermal patches so as to achieve systemic administration.

Suitable injectable solutions include intravenous subcutaneous and intramuscular injectable solutions. The somatotropin may also be administered in the form of an infusion solution or as a nasal inhalation or spray.

Each formulation according to the present invention may additionally comprise inert constituents including pharmaceutically-acceptable carriers, diluents, fillers, salts, and other materials well-known in the art, the selection of which depends upon the dosage form utilized, the particular purpose to be achieved according to the determination of the ordinarily skilled artisan in the field and the properties of such additives. Examples of carriers and diluents include carbohydrates and lipids including without limitation phosphatidyl choline, phosphatidyl serine, phosphatidyl ethanolamine, triglycerides, tocopherol, retinoic acid and cyclodextrins and its derivatives.

The active ingredient of the present invention may additionally be incorporated into transdermal delivery systems or liposomes. Such liposomes may also comprise other active agents, e.g. specific agents to direct the Tableliposome to its desired site, such as an antibody to a particular type of brain cell.

The pharmaceutical preparations of the present invention are manufactured in a manner which is itself known, for example, by means of conventional mixing, granulating, dragee-making, dissolving, or lyophilizing processes. Thus, pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipients, optionally grinding the resulting mixture and processing the mixture of granules, after adding suitable auxiliaries, if desired or necessary, to obtain tablets or dragee cores.

Somatotropin in a pharmaceutically acceptable carrier may be used alone or in combination with other medicaments. Medicaments are considered to be provided "in combination" with one another to form a pharmaceutical combination if they are provided to the patient concurrently or if the time between the administration of each medicament is such as to permit an overlap of biological activity. The two or more agents may be administered by separate routes such as providing somatotropin by injection and providing 1,2,3,4-tetrahydro-9-aminoacridine (THA) orally.

The list of other chemicals which may be used in combination in the present invention to enhance learning and memory is long. Any of the known or suspected agents which affect such a biological function may be used. Examples include: physostigmine, THA, propranolol, ACTH, its variants and analogues, vasopressin, vasodialators, amphetamine compounds and derivatives, and a wide variety of agents which alter the concentrations of neurotransmitters in the brain. These chemicals may be used with the ordinary pharmaceutical carriers useful for that chemical and are not necessarily limited to the carriers to be used with somatotropin. Agents may also be used which would diminish an adverse effect of somatotropin.

The human somatotropin may be formulated in a pharmaceutical carrier so that it is slowly released or released at a later time. This delayed or sustained release function may also be applied simultaneously or separately to any other medicament being administered concurrently as well.

Compositions within the scope of this invention include all compositions wherein the somatotropin is provided in an amount effective to achieve its intended purpose. While individual needs vary, determination of optimal ranges of effective amounts of each component is within the skill of the art. Typical dosages comprise 0.05 to 1.5 mg/kg body weight per day.

The preferred dosage will depend on the individual and the nature of the condition being treated. The dose should be high enough to produce the desired effect, but low enough so as not to produce acromegalic-like conditions in individuals with closed ephyses (or treatment of such patients may be avoided altogether). It will be appreciated that the unit content of active ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount provided that the effective amount can be reached by administration of a plurality of doses. It may be advantageous to administer GH in the form of a timed-released capsule prior to bedtime in order to mimic the prominent nocturnal spike of endogenous GH.

A useful starting point for determining the proper dose is the recommended dose of human somatotropin for stimulation of growth. Eli Lilly's HUMATROPE dosage form is 5 mg somatotropin (13 IU or 255 pmoles) per 5 ml vial. Eli Lilly recommends a dosage of up to 0.6 mg/kg (0.16 IU/ks) of body weight administered 3 times a week by subcutaneous or intramuscular injection. Genentech's PROTROPIN dosage form

is similar to HUMATROPE. However, Genentech's maximum recommended dosage is 0.1 mg/kg (0.26I6/kg) body weight. If this dose is effective, it may be reduced in linear, logarithmic or other increments until the minimum effective dose for improvement of learning or memory becomes apparent. If this dose is ineffective for the purpose of the present invention, it may be increased in linear, logarithmic or other increments until the minimum effective dose is reached. The effect must of course be weighed against any adverse reactions, such as hypoglycemia, hyperglycemia, and acromegaly. It may be possible to mitigate adverse effects of high dosages with a second drug.

A number of diseases or other medical conditions have or may have mental impairment as a primary problem or a symptom of a condition. Such diseases include AIDS dementia, stroke, Huntington's disease, ALS (Lou Gehrig's disease), Alzheimer's disease, drug or drug interaction induced dementia or physical injury. The symptoms may be alleviated using the composition of the present invention. The invention may also be useful for normal people wanting to improve their learning and memory for school, the work place, etc.

The recipients are not limited to humans. It may be advantageous to have pet, sport or agricultural animals with improved learning and memory. One such example are animals which help the blind and the disabled. The present invention is also particularly useful in veterinary medicine for treating an injured or diseased animal.

EXAMPLE

The experiments involved four groups of mice. In the first group 12 mice were used in which the PEPCK-HGH gene was expressed under basal conditions, i.e., without induction. The second group was 12 age and sex matched non-

transgenic litter mates which lack the hGH gene, as controls. The third group was 8 mice in which the MT-HGH gene was expressed, again under basal conditions. The fourth group was 8 sex and age matched litter mates in which the gene was not expressed. Serum hGH has been determined in large numbers of MT/hGH and PK/hGH transgenic mice; typical values are 7-15 ng/ml and 130 - 150 ng/ml, respectively. However, serum levels were not measured in the animals used in this experiment since stress is associated with bleeding and can affect both mental function and hGH serum concentrations. Serum levels of mouse GH would be low since somatotropic activity is suppressed in hGH transgenic animals. The hGH transgenic mice were produced by a procedure analogous to that described for rabbit beta globin. See Wagner, et al., PNAS, 78:6376 (1981); and cf. Palmiter, et al., Science, 222:809 (1983) (MT-hGH); Hanson, W088/10304 (PEPCK-bGH).

The mice were housed two to a cage initially provided with food and water ad lib. The mice were maintained on a normal day/night cycle with all procedures carried out during the day. One mouse was lost in the PK experimental group on run day 5 and another in that group on run day 10 of the study. These subjects were not included in the analysis of the data.

Learning and memory tests were carried out in a simple T-maze. The maze consisted of a start box leading to a main stem runway which connected to right and left arms which branched at right angles to the stem. The right and left arms ended in goal boxes which contained a goal cup. The goal cup was a metal cube 1 cm. on a side with a small depression in the top in which milk was placed. All runways, start and goal boxes were covered with transparent tops which could be removed for access to the animal. The start box and goal boxes could be separated from the runways

by clear doors which slid across the runways. The start and goal boxes were 15 cm long, the main runway 18 cm and arm runways each 23 cm long. The walls of the main runway were gray, the right arm walls white and the left black.

Three days prior to the start of the learning and memory trials, the water available to the mice was replaced with a 50/50 mixture of water and condensed milk to acclimate the animals to the milk mixture. This milk mixture was used in all reinforcement of maze running. Approximately 10-12 hours prior to the first trial, the milk bottles were removed from the animal cages, with food continuously available. Following each day's trials, the milk mixture was replaced on the cages until about 10 hours prior to the next run. Prior to training, each animal was randomly assigned to either right or left turn correct, with the stipulation that equal numbers of genetically altered and controls must be assigned to right and left turns.

The first day of training consisted of placing the animal in the open maze for 3 to 4 minutes with the top closed. The animal was then placed in the correct closed goal box which contained milk in the goal cup for two minutes, then in the closed start box for 2 min., with this goal box-start box sequence repeated 5 times. The animal was then allowed to explore the open maze for another 4 min. On the next day (first training trail day) the animal was placed in the closed start box and the door opened. The animal was allowed to run the maze until it entered a goal box far enough for the door to be closed. The trial was counted as correct if the goal box was the one containing milk and incorrect if the animal entered the empty goal box. If 2 min. elapsed without the animal entering a goal box, the trial was scored as a no goal trial. If the animal did not leave the start box, the animal was given a no trial score and that trial was not counted in the total trial count. Scores were computed as number of correct trials divided by total run

trials (trials on which a goal box was entered plus trials run but no goal box entered).

Between each trial, the entire maze floor was swabbed with 50% ethanol to eliminate odor cues and to remove any feces and urine. Training was continued for the predetermined number of training days, with no training on weekends.

The PK groups were given additional training trials 27 days following the original acquisition trials. These animals were placed on deprivation and given an additional 6 days of training as described above. These trials were designed to test retention and any further learning following the period of no maze activity.

The results of the acquisition and retention trials of the PK experimental and control groups are shown in Figures 1 and 2. As can be seen from Figure 1, the two groups of subjects had essentially random performance in correct verses incorrect turns during the first two days of training (20 trials). The performance then began to shift to more correct turns for both groups, but increased faster for the PK-transgene group as compared to the controls. The group performance essentially reached asymptotic performance on about day 9 and remained fairly stable thereafter. Figure 2 shows the performance of the two groups over the 6 retention training days. It can be seen that the PK-transgene group performed consistently higher over the period and there were essentially no further increases in performance over trials in either group. Analysis of variance showed that there was a significant group difference ($p<.0384$, df 1, 20) and a significant trials effect ($p<.0001$, df 13, 260) over days of training, but no interaction between groups and trials. These results suggest that both groups showed increasing performance over training, but that the PK-transgene group showed a higher level of performance. Analysis of variance of the retention training trials showed only a significant groups effect ($p<.001$, df 1, 16). This supported the impression that the PK

group performed consistently better than the controls over the 6 days and that neither group's performance changed over the trials.

Figure 3 shows the results of the acquisition trials for the MT experimental and control groups. Again, the data suggest an increase in performance for both groups over training, and a superior performance for the MT group. An analysis of variance supported the increasing trend for the two groups with a significant trials effect ($p<.0001$, df 13, 182), but did not show a significant groups effect. However, a two-tailed dependent t-test of the group performance showed a highly significant effect ($p<.0001$, df 13) when the two groups were compared directly. The lack of a significant effect in the analysis of variance is undoubtedly due to a smaller number of subjects and a greater variability seen in these groups. These results suggest that the MT animals also learned the maze task better than did control subjects. No retention trials were run with these subjects.

These results indicate the PK- and MT-transgene animals learned to perform the single turn T-maze task better than did the control subjects. In addition, while both PK and control animals retained the task over 21 days, the PK animals retained their superior performance while the controls showed an asymptotic performance at a lower level. This suggests that the PK animals had a superior ability to learn the task as well as a faster acquisition of the correct performance.

The present data are primarily from a procedural task, although the maze task has components of discrimination and spatial learning as well. These components in the animal model may well correspond to elements of both semantic and episodic memory in humans.

The foregoing description of the specific embodiments reveal the general nature of the invention so that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing

from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

All references mentioned in this application are incorporated by reference.

Table 1

	occurrences in wt HGH	# of Differences (1)
A	7	28
C	4	0
D	11	50
E	14	58
F	12	54
G	8	51
H	3	18
I	8	34
K	9	45
L	26	63
M	2	9
N	9	53
P	8	29
Q	13	58
R	10	60
S	19	106
T	10	63
V	7	41
Y	8	42
-	4	33
W	1	0

Note (1): total number of differences between wt HGH and seven other mammalian GHs, for all appearances of indicated amino acid in wt HGH.

Table 2

	A	C	D	E	F	G	H	I	K	L
A				2	1	1		1		
C										
D	6			11	1	2	9		3	
E			14		3	9	4		8	
F	5					2	1	2	1	13
G	10			14						
H										3
I	2				4					3
K			1	8		4				
L				4	10	1		4	6	
M								4		5
N			10			8	1	11		
P	3		2	2	7			2		3
Q			1	4		1	1		19	16
R	4		1	5		3	10		15	1
S	9		8	1		1		4	2	8
T	29		3	1		1		5	2	7
V	8					5		2		3
Y	4			1	12					5
-	3			2	6	1				1

Table 2 (Cont'd)

	M	N	P	Q	R	S	T	V	Y	-
A		1				5	10	3		4
C										
D		4	2		2	4	1		2	3
E	1	3		4	5	2	3	1	1	
F		7		7		2	1		11	2
G		1	1	1	10	5	7	2		
H				9	5					1
I	5			3		1	3	13		
K				4	14	8				9
L	2	11	1	1	5	7		5	3	3
M										
N				3	5	5		5		5
P						4	4	1		1
Q		2	1		6	7				
R						14	4	1	1	1
S	5	8		8	9		14	10	6	13
T	1		1	2		4		2	3	2
V	7		3			7	3			3
Y			4	3		6		1		6
-					3	3		1		13

What is claimed is:

1. A method for improving the learning or memory functions of an individual comprising administering an effective amount of a human somatotropin.

2. The method of claim 1 wherein the individual is a human.

3. The method of claim 2 wherein the individual has a condition resulting in a learning or memory deficit.

4. The method of claim 3 in which the deficit is attributable to age.

5. The method of claim 3 in which the deficit is attributable to Alzheimer's disease.

6. The method of claim 3 in which the deficit is attributable to injury.

7. The method of claim 1 in which the individual has normal learning and memory.

8. A method for determining whether a polypeptide will improve the learning or memory functions in an individual comprising:

preparing a transgenic animal with a transgene encoding said polypeptide wherein expression of said polypeptide in said transgenic animal is regulatable,

inducing expression of said polypeptide in said transgenic animal,

having said transgenic animal with induced expression of said polypeptide perform a test measuring learning or memory function, and,

comparing the test measuring learning or memory results from the animal with induced expression to (a) a control animal which does not produce the same concentrations of the polypeptide or (b) to said animal prior to induction of expression.

9. The method of claim 13 wherein said polypeptide is not a polypeptide naturally occurring in the animal.

10. The method of claim 13 wherein expression of said polypeptide is regulatable by means which does not stress the animal.

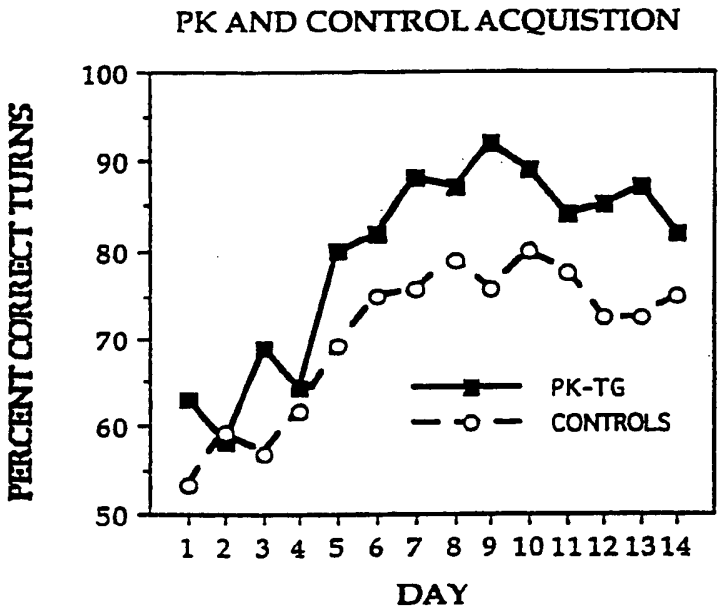
PK AND CONTROL ACQUISTION
PERCENT CORRECT TURNS
100
90
80
70
60
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAY
PK-TG
CONTROLS

Figure 1

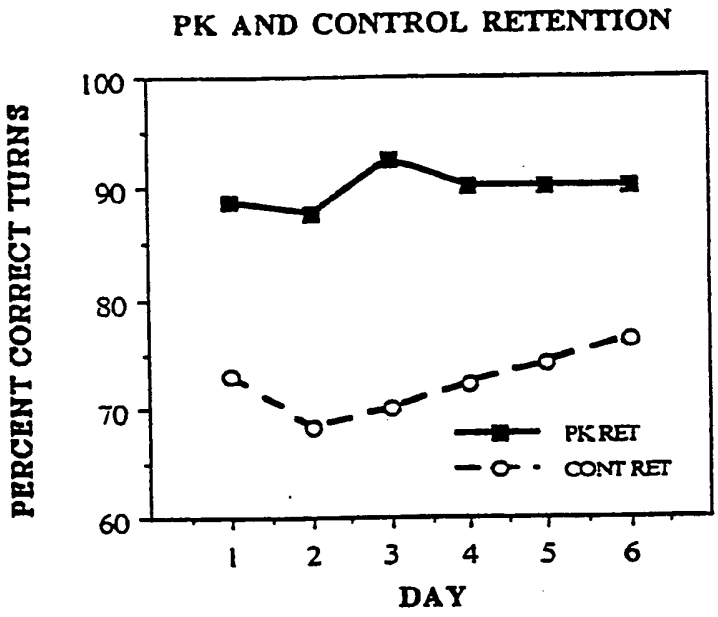
PK AND CONTROL RETENTION
PERCENT CORRECT TURNS
100
90
80
70
60
1
2
3
4
5
6
DAY
PK RET
CONT RET

Figure 2

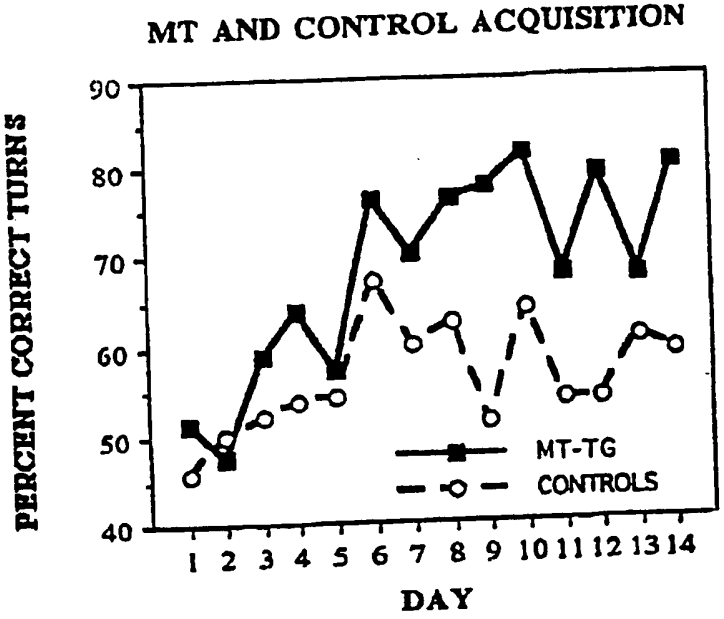
MT AND CONTROL ACQUISITION
PERCENT CORRECT TURNS
90
80
70
60
50
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAY
MT-TG
CONTROLS

Figure 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/03005

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/36 A01K67/027 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 324 037 (AROONSAKUL) 19 July 1989 see the whole document ---	1
A	EP,A,0 326 381 (LABORATORIOS SERONO) 2 August 1989 see the whole document ---	1
A	WO,A,90 05185 (L'UNIVERSITE DE L'ETAT A LIEGE) 17 May 1990 see the whole document ---	1
A	WO,A,92 06187 (THE UPJHON COMPANY) 16 April 1992 see the whole document -----	8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1994	14.07.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03005

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1-7 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US 94/03005

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0324037	19-07-89	US-A-	4898856	06-02-90
		US-A-	4898857	06-02-90
		US-A-	4897389	30-01-90
		US-A-	4727041	23-02-88
EP-A-0326381	02-08-89	US-A-	4939124	03-07-90
		US-A-	5089472	18-02-92
		AU-A-	2870089	27-07-89
		JP-A-	2042025	13-02-90
WO-A-9005185	17-05-90	EP-A-	0441889	21-08-91
		JP-T-	4503154	11-06-92
WO-A-9206187	16-04-92	AU-A-	8768191	28-04-92
		EP-A-	0550675	14-07-93
		JP-T-	6502071	10-03-94